EXHIBIT

A

The Effect of LMS-611 and Comparator Controls on Biological Gels *In Vitro*

Surfactants comprise of natural phospholipids and can reduce sputum adhesivity (Hite 2002) (Boucher 2007). Liposomes are micro-vesicle excipients used in drug delivery and comprise of phospholipids and cholesterol. The effect of the surfactants Survanta (25mg/ml) and Curosurf (80mg/ml) and commercially available, unilamellar liposomes, L4031 (L-a-phosphatidylcholine, sterylamine and cholesterol and L4395 (L-a-phosphatidylcholine b-oleoyl-glpalmitoyl, L-a-phosphatidyl-DL-glycerol, dioleoyl and cholesterol) were tested for effect on biological gels using the inclined plane method. The surfactants little effect on the adherence of the biological gels to the inclined plane. Although, Curosurf, on repeated administration, slowly mobilised DNA which travelled the distance within the time frame permitted. The unilamellar liposomes had a slight effect on the adherence of the gels to the inclined plane. LMS-611 (5mg/ml) had an immediate effect significantly reducing the adherence of mucin, alginate and DNA to the inclined plane (Figure 10).

Figure 10. The effect of surfactants, liposomes and LMS-611 on the adherence of different biological gels to an inclined plane.

Reversibility of Effect

LMS-611 (19.8mg/ml) or saline (Control) were added to mucin (5%) in the presence of protamine (10mg/ml) and incubated for 3 minutes at room temperature. Protamine was included to help aggregate the LMS-611. The adherence of the samples was determined using the inclined plane test (Pre-centrifugation sample). The samples were then centrifuged (2300rpm for 20mins). The adherence of the samples was then re-tested (Post-centrifugation sample). Adherence was measured as the time for the sample to travel 5cm under the influence of gravity down an inclined plane.

The time for the Control pre-centrifugation sample to travel the distance was not significantly different from that of the post-centrifugation sample, 112.8 seconds and 124 seconds respectively. In contrast, the

LMS-611 treated sample took on average in 17.3 seconds to travel the distance prior to centrifugation and 155 seconds post-centrifugation. The property of adherence was restored to the mucin by the separation of LMS-611 from the mucin by centrifugation.

As the action of LMS-611 to reduce the adherence of biological samples can be reversed by centrifugation the mechanism of action of LMS-611 has been shown to be biophysical and not pharmacological in nature.

Dynamic Mixing Process

As the biological gels tested were transparent, direct light microscopic observation of the mixing of LMS-611 with the gel could be observed. Two droplets (5l), one mucin (5%) and the other of LMS-611 (19.8mg/ml), were carefully pipetted side by side with their edges almost touching on a glass slide on the stage of a light microscope. Dark background illumination sharpened the profiles of the materials under observation otherwise no stains or contrast media were used. The expansion of the slowly spreading LMS-611 on making contact with the mucin suddenly, rapidly and forcefully imploded into the mucin droplet (Figure 11). LMS-611 was seen to move in numerous streams progressively penetrating deeper into the gel, separating the gel "fibres" into elongated cords and islands. Movement continued for several minutes. By focusing at different planes of the section, it was confirmed that all levels of the gels were permeated, while confocal microscopy confirmed this by 3D visualisation. The same dynamic-mixing phenomenon was observed when LMS-611 came in contact with a droplet of DNA or alginate. The mixing phenomenon was not seen when a droplet of saline (0.9% NaCl) came in contact with these gels. (See Figure 11)

The Effect of LMS-611 on the Rheological Properties of Biological Gels

A series of studies were performed to quantify the effect of LMS-611 on the rheological properties of ex-vivo samples of sputum from subjects with cystic fibrosis. The effect of LMS-611 on the rheological properties of alginate was also studied. The rheological properties of materials are characterised by the following parameters:

Elasticity: The elastic solid is an energy-storing device. On application of a force, there is displacement of the structure and energy is stored within it. When the force is removed, the energy is released and the structure reverts to its original state. The higher the elasticity or dynamic storage modulus (G) of a substance the greater the amount of energy that can be stored within it

for a given degree of displacement, which implies a highly developed interlinked structure.

Viscosity: The higher the viscosity or dynamic loss modulus (G) of a substance the greater the force required to create a given degree of displacement which implies a more solid structure, whereas a low viscosity indicates a more liquid structure.

Dynamic complex modulus (G*) or rigidity factor is a measure of both elasticity and viscosity and represents the overall stress to strain ratio in a dynamic experiment. A high dynamic complex modulus indicates a very rigid structure.

The linear visco-elastic range of CF sputum samples lay between 0.1 and 10 Pa. Frequency sweeps were therefore carried out in this region.

Amplitude and frequency sweeps are oscillatory techniques where a sinusoidal stress or strain is applied to the sample. While sample structure is maintained the complex modulus (G*) is roughly constant. The visco-elastic properties of a sample are defined in terms of their elastic modulus (G) and viscous modulus (G), which are measured by the frequency sweep.

Sputum was collected with informed consent from cystic fibrosis subjects undergoing physiotherapy. Sputum donors, both female and male, 15 years of age or above, were recruited. Most subjects were being treated with the mucolytic, Pulmozyme. Samples were therefore considered to be Pulmozyme positive unless it could be verified that subjects had not received Pulmozyme in the last 3 months. Sputum samples were excluded if the subject had received nebulised Pulmozyme within 4 hours of sputum production. Sputum samples were aliquoted (400mg), sprayed with 2 ml of either LMS-611 (19.8mg/ml), saline (0.9% NaCl) or Pulmozyme (1mg/ml) and incubated at 24oC for 1 hour with gentle rotation. The rheological properties of the sputum were then measured using a Bholin Gemini Rheometer (Malvern Instruments) with a parallel plate (20cm) measuring geometry (200 m gap) and Peltier plate, to maintain the temperature at 24oC.

The visco-elasticity of the CF sputum samples was, however, found to be highly variable both inter- and intra-sample making data analysis and comparison difficult.

In excess of 50 sputum samples have been treated with LMS-611 and LMS-611 was found to reduce the visco-elastic properties of the sputum from CF subjects in all instances. LMS-611 was found to be more potent than normal saline (Table 1, Figures 12 and 13),

significantly more potent than the surfactant Survanta (Table 1) and of a similar potency to Pulmozyme (Table 1 and Figure 14) in reducing the visco-elasticity of CF sputum samples.

Group	n=	Avg. Elastic modulus (G)	Avg. Viscosity modulus (G)	Avg. Complex modulus (G*)
Saline (0.7% NaCl)	9	150 +/- 188	81.8 +/- 149	150.6 +/- 176.5
LMS-611 (5mg/ml)	9	44 +/- 61.7	16.9 +/- 21.0	47.2 +/- 65.2

Group	n=	Avg. Elastic modulus (G)	Avg. Viscosity modulus (G)	Avg. Complex modulus (G*)
Pulmozyme (1mg/ml, 1000 Units)	7	11.4 +/- 9.1	6.9 +/- 4.6	13.4 +/- 9.6
LMS-611 (5mg/ml)	7	13.3 +/- 9.8	5.4 +/- 3.6	14.4 +/- 10.0

Group	n=	Avg. Elastic modulus (G)	Avg. Viscosity modulus (G)	Avg. Complex modulus (G*)
Survanta (25 mg/ml)	2	32 +/- 5.7	19.0 +/- 1.4	37.5 +/- 3.5
LMS-611 (5 mg/ml)	2	18 +/- 0.0**	7.5 +/- 0.7**	19.0 +/- 0.0**

Key: Avg. = average; +/- Standard deviation; ** = P-value <0.05

Table 1. The effect of LMS-611, Saline, Pulmozyme and Survanta on the rheological properties of CF sputum.

Figure 12. The effect of Saline (0.9% NaCl) and LMS-611 (5mg/ml), compared to the No-Treatment Control (NTC), on the visco-elastic moduli of an ex -vivo sputum sample from a CF subject (Pulmozyme positive).

Figure 13. The effect of Saline (0.9% NaCl) and LMS-611 (5mg/ml) on the visco-elastic modulus of an ex-vivo sputum sample from a CF subject (Pulmozyme positive).

Figure 14. The effect of LMS-611 (5mg/ml) and Pulmozyme (1mg/ml, 1000 Units) on the visco-elastic moduli of an ex-vivo sputum sample from a CF subject (Pulmozyme negative).

LMS-611 was also found to reduce the rheological properties of alginate.
This effect was more profound than saline, which also reduced the visco-elastic properties of the alginate (Figure 15).

Figure 15. Effect of LMS-611 (19.8mg/ml) and Saline (0.9% NaCl) on the rheological properties of alginate (3500cps).

In the presence of $CaCl_2$ the alginate becomes more visco-elastic, as a result of additional crosslinking of the gel. LMS-611 significantly reduced by 94% the visco-elastic properties of the CaCl2/alginate gel compared to a 64% reduction seen following exposure to saline (0.9% NaCl).

<u>The Effect of LMS-611 on the Surface Tension and Contact Angle of Biological Gels</u>

In addition to the rheological properties of mucus, wettability is also an important factor in the interaction between mucus and the cilia and respiratory epithelial surface. Wettability is the tendency of a biological fluid to spread when deposited on a solid plane surface. The degree of wettability is determined by the contact angle between the tangent to the air liquid interface and the horizontal at the triple point where the three phases meet. Studies were undertaken to compare the effects of LMS-611 on the surface tension and the contact angle of carboxymethylcellulose (3500cps), mucin (2.5%; from bovine submaxillary glands), DNA (1%; polymeric form from salmon testes) and CF sputum obtained from patients following physiotherapy. The sputa were divided into 400mg amounts to which was added 200 l of LMS-611 or Pulmozyme and incubated at 37°C for 2 hours before measurement using a CAM101

(Krauss). The contact angle and surface tension of untreated CF sputum cannot be measured due to its highly visco-elastic nature. The effects of LMS-611 and Pulmozyme on the contact angle and surface tension of CF sputum are shown in Table 2.

LMS-611 produced a significantly greater reduction in the wettability and surface tension of CF sputum when compared to Pulmozyme (Table 2). LMS-611 also significantly reduced the wettability and surface tension of carboxymethylcellulose, mucin and DNA (data not shown). The LMS-611 dose-response curve for the reduction in the surface tension of carboxymethylcellulose is shown in Figure 16.

Group	n=	Avg. Contact Angle (Degrees)	Avg. Surface tension (mN/m)
LMS-611 (20mg/ml)	5	66.1o +/- 5.2**	35.3 +/- 2.6**
LMS-611 (5mg/ml)	5	80.5o +/- 2.6**	45.3 +/- 2.1**
Pulmozyme (1000 units)	5	89.7o +/- 1.9	52.1 +/- 3.5

Key: Avg. = average; +/- Standard deviation; ** = P-value <0.05

Table 2. The effect of LMS-611 and Pulmozyme on the wettability (contact angle) and surface tension of ex-vivo CF sputum samples.

Figure 16. The effect of LMS-611 on the surface tension of carboxymethylcellulose.

Micro-reservoir Fluid Regulation

The inhalation of the LMS-611 multi-lamellar micro-vesicles which are hydrated in physiological saline, will, in theory, occupy the periciliary layer of the ASL. Here the micro-vesicles will replace and adopt the micro-reservoir fluid-regulatory properties normally provided by endogenous lamellar bodies and restore the homeostatic maintenance of normo-volaemia of the airways surface fluid of cystic fibrosis patients.

The claims of re-hydration and fluid regulation of the ASL for LMS-611 are supported by the understanding of the structure and function of endogenous lamellar bodies in the ASL and peritoneum (Figures 2, 3 and 4) (Dobbie and Anderson 1996). Here the lamellar bodies, being composed of phospholipid bilayers, appear to act as a series of semi-permeable membranes facilitating passage of water in and out of each and every vesicle consequent on solute concentration differences in individual vesicles of the surfactant foam filling the perciliary and peritoneal liquid layers.

The invention of liposomes has its origin in the search for an experimental model which closely mimicked the physiology of cell membranes. Thus the initial development of conventional or classical liposomes led to advances in our understanding of the physical chemistry of biological membranes (phospholipid bilayers). This information has proved to be most helpful, in that transmission electron microscopy of different types of liposomes is available for use in interpreting the profiles of vesicles derived from extra-alveolar lamellar bodies. Dependent on composition and method of preparation, conventional liposomes exhibit a heterogeneous vesicular morphology.

Different types of compartments can occur in multilamellar vesicles (MLVs). Unprocessed MLVs often have non-uniform distributions of the solute entrapped between concentric bilayers (Figure 17). This is in contrast to stable pluri-lamellar vesicles where solute entrapment is evenly distributed throughout the aqueous compartments. In multivesicular liposomes the compartments are contiguous, but not concentric (New 1997).

Transmission electron microscopy of the periciliary layer in bronchial biopsies and the microvillus layer of serous cavities, for example, peritoneum, exhibits vesicular shapes whose morphology is similar to that found in simple conventional liposomes. These correlations are informative with respect to the fact that all of these vesicles, manufactured or natural, have a prime ability to act as semi-permeable membranes. Because phospholipid membranes are semi-permeable, a concentration difference of solute between one side of the membrane and the other can generate an osmotic gradient which will lead to accumulation of water molecules, being the fastest diffusion species on one side.

In the case of a high concentration of solute entrapped inside liposomes, bathed in low concentration buffer outside, the liposomes will swell up as the internal volume of water increases to such an extent that the area of the membrane is considerably increased, with the spacing between adjacent phospholipid molecules being correspondingly widened. Under these conditions an increased rate of leakage through the membrane can occur for solutes of molecular size equivalent to or smaller than glucose. In some cases however, the gradient generated may be sufficient to rupture the membrane completely, causing the loss of the total contents of the liposome into the bulk aqueous phase, before re-sealing again (Lasic 1995).

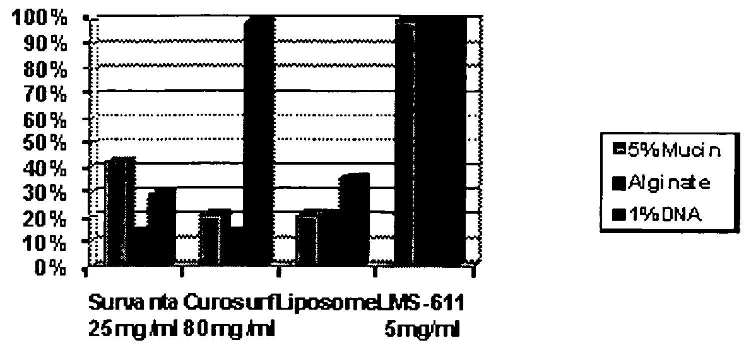

Figure 10. The effect of surfactants, liposomes and LMS-611 on the adherence of different biological gels to an inclined plane.

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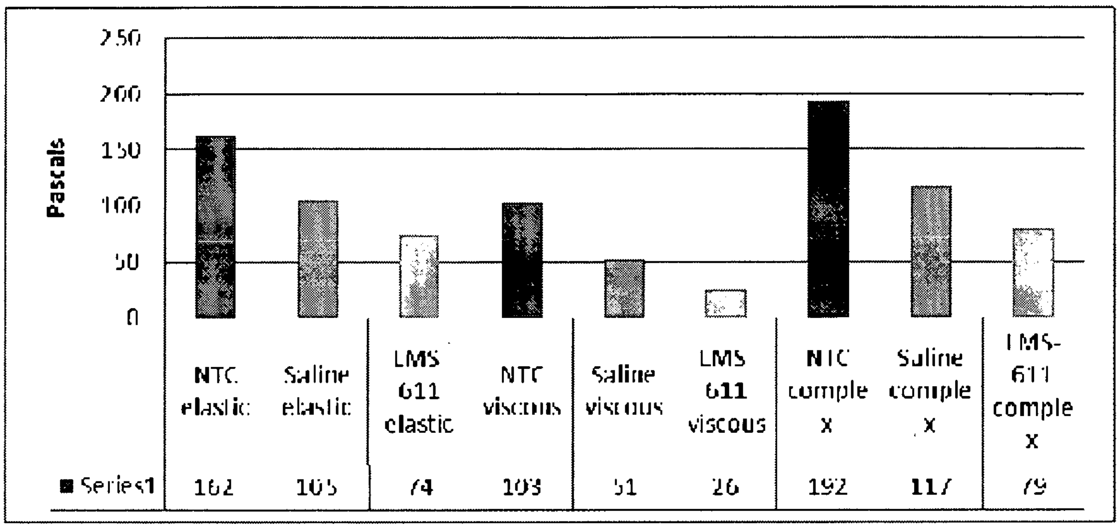

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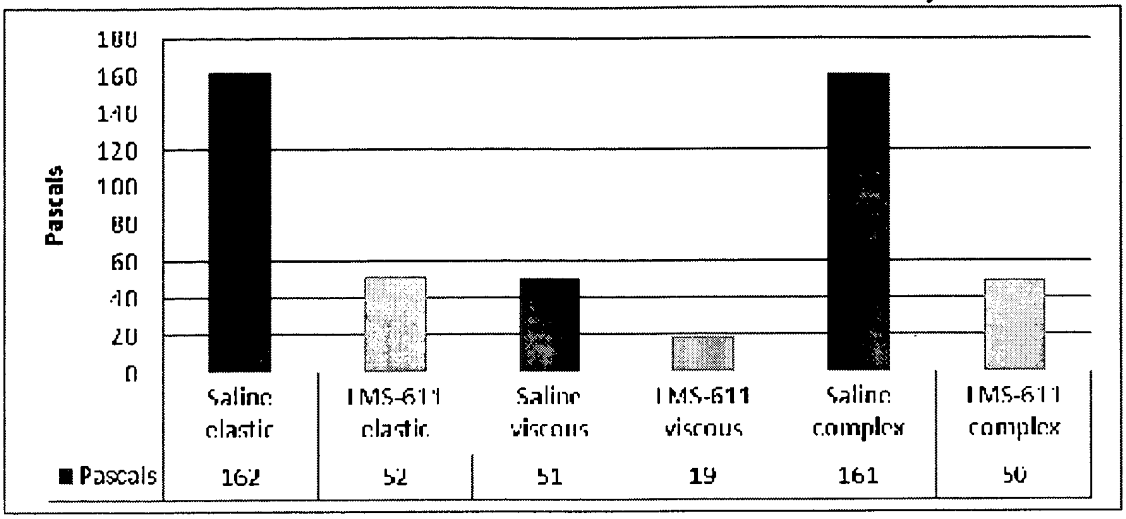

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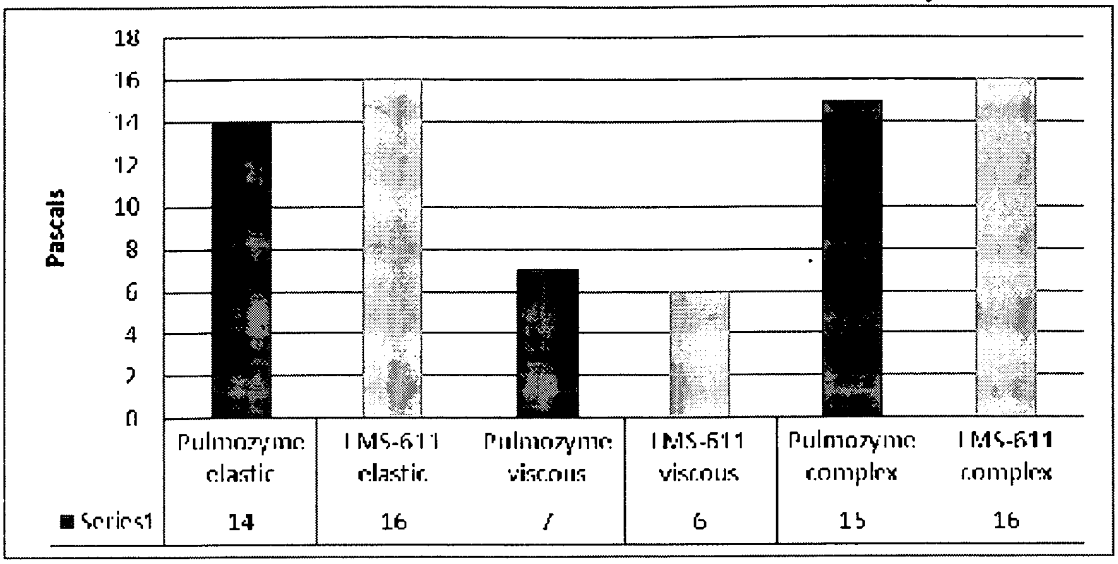

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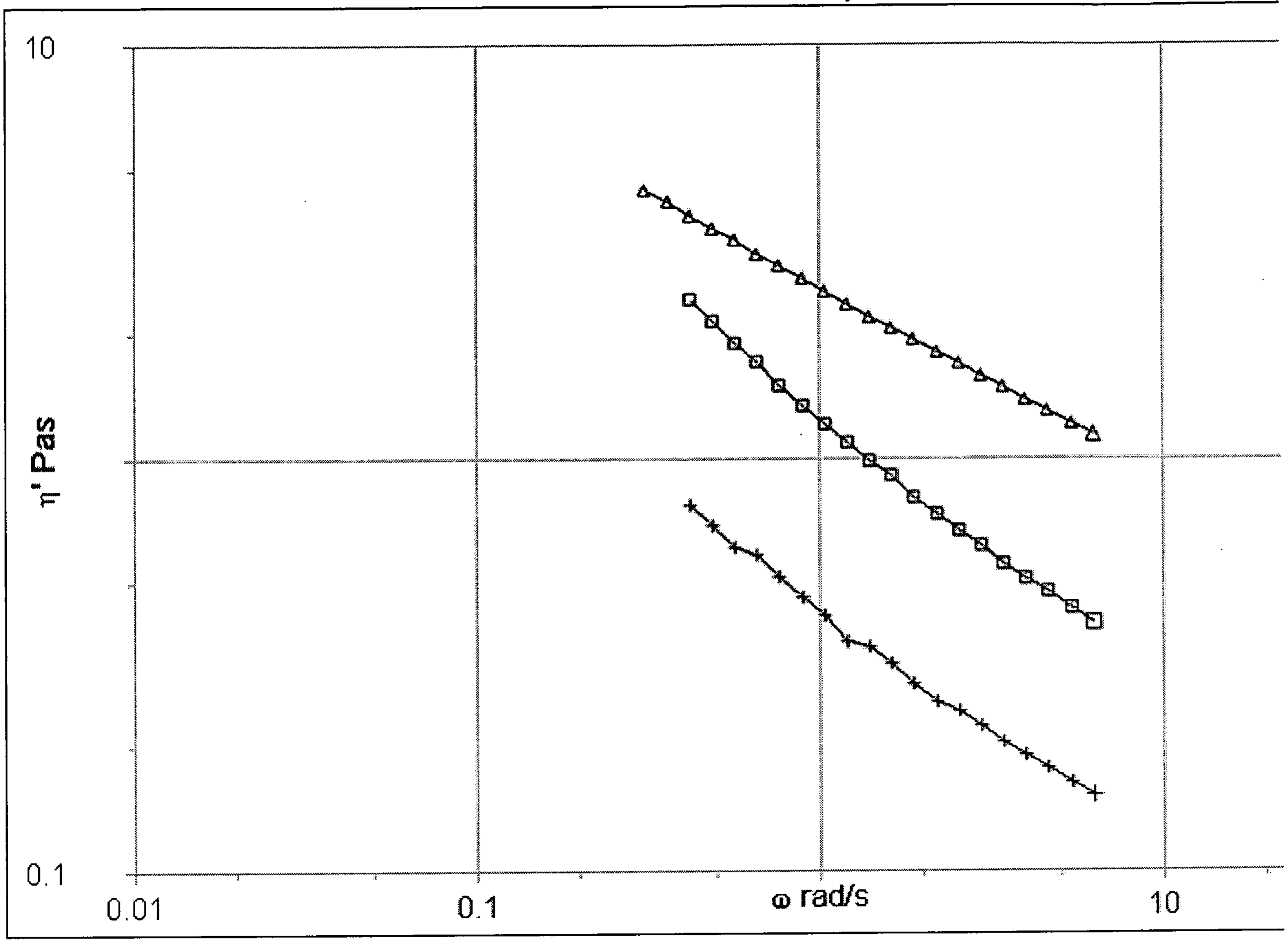

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Table 2. The effect of LMS-611 and Pulmozyme on the wettability (contact angle) and surface tension of *ex-vivo* CF sputum samples.

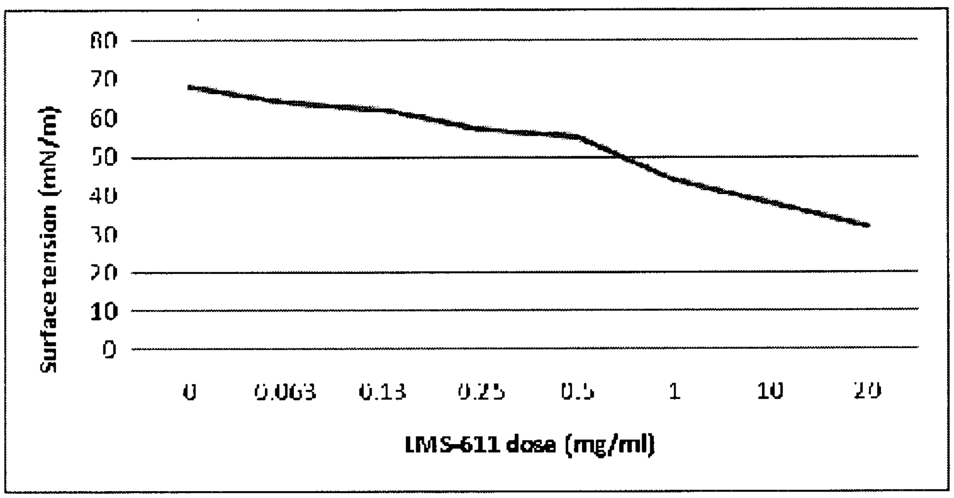

Figure 16. The effect of LMS-611 on the surface tension of carboxymethylcellulose.